Potential Antitumor-Promoting Diterpenoids from the Stem Bark of *Picea glehni*

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A novel rearranged labdane-type diterpenoid, 19(4→3)*abeo*-8α,13(*S*)-epoxylabda-4(18),14-diene (**1**), and two new abietane-type diterpenoids, 19-*nor*-abieta-4(18),8,11,13-tetraen-7-one (**2**) and 12-hydroxydehydroabietic acid (**3**) were isolated from the stem bark of *Picea glehni*, together with seven known diterpenoids—13-epimanoyl oxide (**4**), dehydroabietic acid (**5**), (11*E*)-14,15-*bisnor*-8α-hydroxy-11-labden-13-one (**6**), abieta-8,11,13-trien-7-one (**7**), 9α,13α-epidioxyabiet-8(14)-en-18-oic acid (**8**), 9,10α-epoxy-9,10-*seco*-abieta-8,11,13-trien-18-oic acid (**9**), and methyl 15-hydroxy-7-oxo-dehydroabietate (**10**). Compounds **5**−**8** and **10** showed potent inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate.

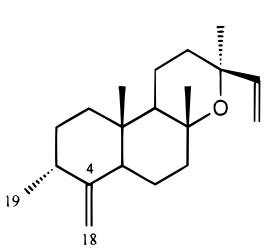
Recently, we published the structures and in vitro antitumor-promoting activities of two new triterpenoids, 3α-methoxyserrat-14-en-21β-yl formate and 24-methylcycloartenone from a chloroform-soluble extract of the stem bark of *Picea glehni* (Fr. Schm.) Masters (Pinaceae).[1] Further careful examination of the extract of the bark has led to the isolation of three new diterpenoids (**1**−**3**) together with seven known diterpenoids (**4**−**10**).

Of these compounds, (11*E*)-14,15-*bisnor*-8α-hydroxy-11-labden-13-one (**6**) and 9α,13α-epidioxyabiet-8(14)-en-18-oic acid (**8**) showed strong inhibitory effects on Epstein−Barr virus early antigen (EBV−EA) induction.

Results and Discussion

The seven known diterpenoids were confirmed as 13-epimanoyl oxide (**4**),[2] dehydroabietic acid (**5**),[3] (11*E*)-14,15-*bisnor*-8α-hydroxy-11-labden-13-one (**6**),[4] abieta-8,11,13-trien-7-one (**7**),[5] 9α,13α-epidioxyabiet-8(14)-en-18-oic acid (**8**),[6] 9,10α-epoxy-9,10-*seco*-abieta-8,11,13-trien-18-oic acid (**9**),[7] and methyl 15-hydroxy-7-oxo-dehydroabietate (**10**),[8] because their physical and spectral data were in good agreement with those already published. Among them, compound **9** has been isolated only once previously, from the leaves of *Larix kaempferi*.[7]

Compound **1** was assigned the molecular formula $C_{20}H_{32}O$ (HREIMS). Its IR spectrum showed absorption bands for a vinyl group and a terminal methylene group. The ^1H and ^{13}C NMR spectra (Table 1) revealed signals for three tertiary methyl groups, one secondary methyl group, two sp^3 fully substituted carbons [δ_C 73.5 (s), 76.0 (s)] compatible with the presence of an ether bridge, an exocyclic methylene group [δ_H 4.40 (t), 4.73 (t), δ_C 105.1 (t), 154.7 (s)], and a mono-substituted double bond [δ_H 6.03 (ddd), 4.92 (dd), 4.98 (dd), δ_C 109.5 (t), 147.7 (d)] and was assigned as a $\Delta^{14,15}$-labdane. The ^1H and ^{13}C NMR spectra resembled those of 13-epimanoyl oxide (**4**), except for the A ring. The gross structure of **1** was determined using the $^1H-^1H$ COSY, NOESY, HMQC, and HMBC techniques. The secondary methyl group was attached at C-3, based on the correlations between H-3 and H-2β and Me-19 in the $^1H-$



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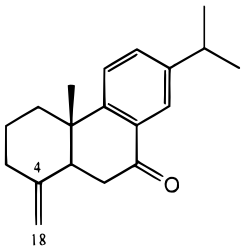

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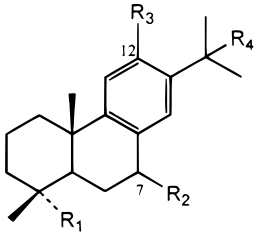
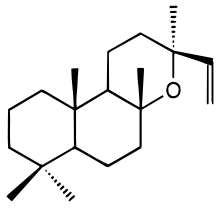


	R_1	R_2	R_3	R_4
3	COOH	H_2	OH	H
5	COOH	H_2	H	H
7	CH_3	=O	H	H
10	COOMe	=O	H	OH



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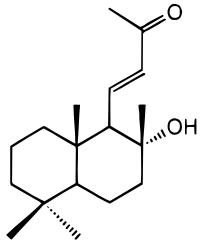

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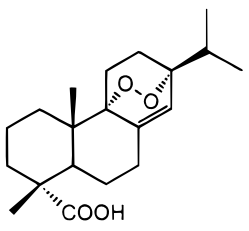

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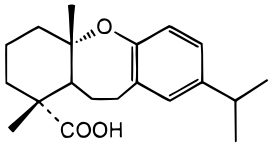

9

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Table 1. ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, and HMBC NMR Data for Compound **1** ($CDCl_3$)

position	δ_C	δ_H	COSY (^{1}H→^{1}H)	HMBC (C→H)
1α	34.0 t	1.30 ddd (13.9, 13.3, 4.6)	1*β*, 2α, 2*β*	3*β*, 9α, 20
1*β*		1.52 m	1α, 2α, 2*β*	
2α	28.5 t	1.37 m	1α, 1*β*, 2*β*	1α, 1*β*, 3*β*, 19
2*β*		1.82 dddd (13.9, 13.7, 5.0, 4.8)	1α, 1*β*, 2α, 3*β*	
3	38.2 d	2.51 m	2*β*, 19	1α, 1*β*, 2α, 2*β*, 18A, 18B
4	154.7 s			2α, 3*β*, 18A, 18B, 19
5α	46.4 d	2.04 br d (10.5)	6α	1*β*, 3*β*, 6*β*, 7α, 7*β*, 18A, 18B, 20
6α	22.5 t	1.43 m	5α, 6*β*, 7α, 7*β*	7α
6*β*		1.50 m	5α, 6α, 7α, 7*β*	
7α	41.7 t	1.44 m	5α, 6α, 7*β*	6*β*, 9α, 17
7*β*		1.78 br dd (8.9, 3.4)	5α, 6α, 6*β*, 7α	
8	76.0 s			6α, 6*β*, 7α, 7*β*, 9α, 11*β*, 17
9α	56.6 d	1.38 m		1α, 7α, 7*β*, 12α, 12*β*, 17, 20
10	39.3 s			1α, 1*β*, 6α, 9α, 20
11α	16.5 t	1.46 m	11*β*, 12α, 12*β*	9α, 12α, 16
11*β*		1.58 m	11α, 12α, 12*β*	
12α	35.0 t	1.52 m	11α, 11*β*, 12*β*	9α, 14, 16
12*β*		2.24 ddd (13.3, 3.0, 3.0)	11α, 11*β*, 12α	
13	73.5 s			11*β*, 15A, 15B, 16
14	147.7 d	6.03 ddd (17.8, 11.0, 1.1)	15A,15B	12*β*, 15A, 15B, 16
15A	109.5 t	4.92 dd (17.8, 1.1)	14, 15B	
15B		4.98 dd (11.0, 1.1)	14, 15A	
16	23.8 q	1.24 s		15
17	32.7 q	1.16 s		7α, 7*β*, 9α
18A	105.1 t	4.40 t (1.6)	5α, 18A	3*β*
18B		4.73 t (1.6)	5α, 18B	
19	20.4 q	1.09 d (7.3)		2α, 2*β*, 3α
20	12.4 q	0.53 s		1α, 1*β*, 5α, 9α

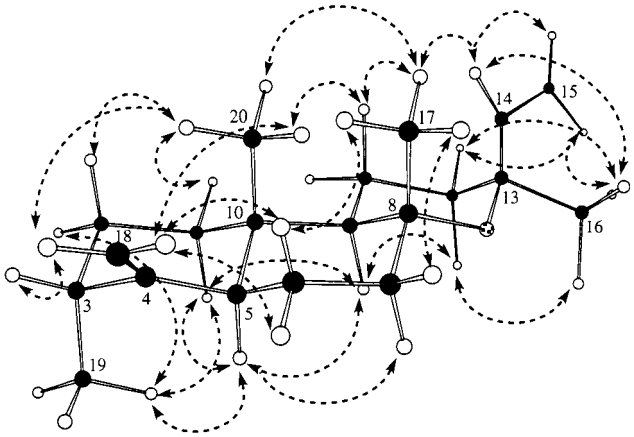

Figure 1.

^{1}H COSY spectrum (Table 1). In the HMBC spectrum (Table 1), C-3 correlated with H-1α, H-1*β*, H-2α, H-2*β*, H-18A, and H-18B; C-4 correlated with H-2α, H-3*β*, H-18A, H-18B, and Me-19; C-18 correlated with H-3*β*, and C-19 correlated with H-2α, H-2*β*, and H-3*β*. The configuration of Me-19 and the conformation of the A ring were determined to be α and a chair form based on the NOESY spectrum. Significant NOE enhancements (Figure 1) were observed for Me-19 with H-1α and H-5α, and H-18A and H-18B with H-6α and Me-20, although no NOE was shown for Me-19 with H-18A or H-18B. The stereochemistry of C-13 was also established by the NOESY spectrum and comparison of the published ^{1}H and ^{13}C NMR chemical shifts of 13-epimanoyl oxide.[2] In the NOESY spectrum, characteristic NOE enhancements (Figure 1) were observed for Me-17 with H-14 and Me-20. Accordingly, the structure of **1** was assigned as 19(4→3)*abeo*-8α,13(*S*)-epoxylabda-4(18),14-diene, with the conformation of the A ring adopting a chair form. Previously, 19(4→3)*abeo*-labdane-type diterpenoids have been isolated from an encrusting Mediterranean sponge *Mycale rotalis*[9] and from the needle oleoresin of *Pinus strobus* (Pinaceae).[10]

Compound **2** was assigned the molecular formula $C_{19}H_{24}O$ (HREIMS). The ^{1}H and ^{13}C NMR spectra (Table 2) revealed signals for a tertiary and two secondary methyl groups, an aromatic ring characteristic of an abieta-8,11,13-triene [δ_H 7.38 (d), 7.42 (dd), 7.94 (d)], an exocyclic methylene group [δ_H 4.64 (d), 4.95 (d); δ_C 108.0 (t), 148.0 (s)], and a conjugated ketone [δ_C 198.4 (s)]. The ^{1}H and ^{13}C NMR spectra resembled those of abieta-8,11,13-trien-7-one (**7**),[6] except for the A ring. The structure of **1** was determined from its ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC NMR spectra. In the HMBC spectrum (Table 2), C-4 correlated with H-3α, H-5α, and H-18A, and C-18 correlated with H-3α and H-5α. Hence, the structure of **2** was assigned as the new compound, 19-*nor*-abieta-4(18),8,11,13-tetraen-7-one. The related compound, 19-*nor*-abieta-4(18),8,11,13-tetraen-7α-ol has been isolated from the bark of *Pinus monticola* Dougl. (Pinaceae).[11]

Compound **3** was assigned the molecular formula $C_{20}H_{28}O_3$ (HREIMS). The IR spectrum showed absorption bands for a hydroxyl group and an aromatic ring. The ^{1}H and ^{13}C NMR spectra (Table 2) revealed signals for two tertiary and two secondary methyl groups, an aromatic ring characteristic of an abieta-8,11,13-triene [δ_H 6.62 (s), 6.83 (s)], a phenolic hydroxyl group [δ_C 150.7 (s)], and a carboxylic acid [δ_C 183.4 (s)]. The ^{1}H and ^{13}C NMR spectra resembled those of 12,15-dihydroxydehydroabietic acid,[12] except for an isopropyl group in **3** rather than a hydroxyisopropyl group. Accordingly, the structure of **3** was assumed to be 12-hydroxydehydroabietic acid, and this was confirmed from its ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC spectra. In the HMBC spectrum (Table 2), C-12 correlated with H-11, H-14, and H-15. Although a considerable number of dehydroabietic acid analogues have been isolated from various natural sources,[13] to the best of our knowledge, compound **3** (12-hydroxydehydroabietic acid) is a new compound.

The inhibitory effects of three labdane-type (**1**, **4**, **6**) and seven abietane-type (**2**, **3**, **5**, **7**−**10**) diterpenoids and the control substance *β*-carotene,[14,15] on EBV−EA activation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA),

Table 2. ^{1}H, ^{13}C, and HMBC Data for Compounds **2** and **3** ($CDCl_3$)

	2				**3**		
position	δ_C	δ_H	HMBC (C→H)	position	δ_C	δ_H	HMBC (C→H)
1α	37.4 t	1.69 ddd (12.4, 12.4, 3.7)	20	1α	37.9 t	1.50 m	3β, 20
1β		2.38 m		1β		2.20 m	
2α	23.6 t	1.88 m	1α, 3α, 3β	2α	18.5 t	1.74 m	1α, 1β, 3α, 3β
2β		1.76 ddd (13.3, 3.7, 3.7)		2β		1.79 m	
3α	36.0 t	2.08 ddd (13.3, 13.0, 5.0)	1α, 18A, 18B	3α	36.6 t	1.79 m	1α, 1β, 2α, 2β, 5α, 19
3β		2.43 ddd (13.0, 4.1, 4.1)		3β		1.70 m	
4	148.0 s		3α, 5α, 18A	4	47.3 s		3β, 5α, 19
5α	46.6 d	2.72 m	6α, 6β, 18A, 18B, 20	5α	44.6 d	2.21 dd (12.5, 2.2)	1β, 3β, 6β, 19, 20
6α	38.0 t	2.68 m		6α	21.9 t	1.52 m	5α, 7α, 7β
6β		2.76 m		6β		1.82 m	
7	198.4 s		5α, 6α	7α	29.2 t	2.83 m	5α, 6α, 6β, 11, 14
8	148.0 s		6α, 6β	7β		2.83 m	
9	151.5 s		12, 14, 20	8	127.1 s		7α, 7β, 11
10	39.2 s		1α, 5α, 6α, 6β, 11, 20	9	147.7 s		5α, 7α, 7β, 11, 14, 20
11	124.8 d	7.38 d (8.0)		10	36.8 s		1β, 2α, 2β, 5α, 6α, 6β, 11, 14, 20
12	132.4 d	7.42 dd (8.0, 2.1)	14, 15	11	110.8 d	6.62 s	14
13	147.0 s		11, 15, 16, 17	12	150.7 s		11, 14, 15
14	125.2 d	7.94 d (2.1)	12, 15	13	131.7 s		11, 15, 16, 17
15	33.6 d	2.94 septet (6.9)	16, 17	14	126.7 d	6.83 s	7α, 7β, 15
16	23.8 q	1.26 d (6.9)	15	15	26.8 d	3.10 septet (6.9)	11, 14, 16, 17
17	23.8 q	1.26 d (6.9)	15	16	22.5 q	1.24 d (6.9)	15, 17
18A	108.0 t	4.64 d (1.1)	3α, 5α	17	22.7 q	1.22 d (6.9)	15, 16
18B		4.95 d (1.1)	5α	18	183.4 s		5α, 19
20	21.3 q	1.11 s		19	16.3 q	1.27 s	3β, 5α
				20	25.0 q	1.20 s	5α

Table 3. Percentage of Epstein−Barr Virus Early Antigen Induction in the Presence of Compounds **1**−**10** with Respect to a Positive Control (100%)[a]

	concentration (mol ratio/TPA)			
compound	1000	500	100	10
1	5.1 (70)	38.8	77.1	100
2	0 (70)	31.6	75.9	96.1
3	2.7 (70)	28.0	66.4	95.8
4	4.3 (70)	35.9	70.4	100
5	0 (70)	30.6	75.0	95.2
6	0 (60)	20.7	68.4	89.3
7	0 (70)	30.2	74.7	95.0
8	0 (70)	24.2	63.7	90.5
9	10.2(60)	52.7	85.0	96.9
10	0 (70)	28.1	72.9	95.0
β-carotene[b]	9 (70)	34	82.0	100

[a] Values represent relative percentages to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells. [b] Positive control substance.

were examined as a preliminary evaluation of their potential antitumor-promoting activities, and the results are shown in Table 3. Thus, compounds **5**−**8** and **10** exhibited potent inhibitory effects (100% inhibition of induction at 1000 mol ratio/TPA, about 70−80% inhibition at 500 mol ratio/TPA, and about 25−40% inhibition at 100 mol ratio/TPA) on EBV−EA induction by TPA.[16,17] The inhibitory effects of these compounds were found to be more potent than that of β-carotene.[14,15] Among these compounds, **6** and **8** exhibited the most potent inhibitory effects on EBV−EA activation (100% inhibition at 1000 mol ratio/TPA, and 80 and 75% inhibition at 500 mol ratio/TPA, and 32 and 36% inhibition at 100 mol ratio/TPA, respectively), and preserved the high viability of the Raji cells. On comparison of the antitumor-promoting activities of **1** and **4** and of **2** and **7**, **4** and **7** showed slightly stronger effects than those of **1** and **2**. It is, therefore, interesting to note that the presence of a geminal dimethyl system at C-4 in compounds of either the labdane or abietane type seems to enhance the resultant antitumor-promoting activity.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-1000 digital polarimeter. UV spectra were recorded using a Hitachi 150-20 specrophotometer. IR spectra were recorded using a Perkin-Elmer 1720X FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively. $CDCl_3$ was used as the solvent and TMS as the internal standard. EIMS were recorded on a Hitachi 4000H double-focusing mass spectrometer (70 eV). Column chromatography was carried out over Si gel (70−230 mesh) and medium-pressure liquid chromatography (MPLC) was carried out with Si gel (230−400 mesh, Merck) and Cosmosil 40C_{18}-PREP (ODS, Nacalai Tesque, Japan). Fractions obtained from column chromatography were monitored by TLC (Si gel 60 HF_{254}). Preparative TLC was carried out on Merck Si gel PF_{254} plates (20 × 20 cm, 0.5 mm thick).

Plant Material. The stem bark of *P. glehni* (Fr. Schm.) Masters was collected in mountainous terrain under the control of National Hokkaido Bureau, Iwamizawa City, Japan, in October 1997. A voucher specimen of *P. glehni* (PG-9710-1) is deposited at the Herbarium of the Department of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

Extraction and Isolation. The extraction and preliminary Si gel column chromatography of the $CHCl_3$ extract of the stem bark of *P. glehni* has been reported,[1] with separation into 13 main fractions (I−XIII). Residue III (3.347 g, fractions 7−9) was rechromatographed over Si gel, Sephadex LH-20, and MPLC (ODS) using CH_3CN to give compounds **1** (4.1 mg), **2** (1.5 mg), **4** (85.0 mg), **7** (4.2 mg), and **10** (4.5 mg). Residue V (69.80 g, fractions 21−33) was purified by preparative TLC ($CHCl_3$) to afford compound **5** (1.03 g), and residue VIII (3.876 g, fractions 71−72) was subjected to Si gel column to give compound **8** (28.0 mg). Residue X (4.768 g, fractions 76−80) was rechromatographed twice over Si gel followed by Sephadex LH-20 to yield compound **3** (4.1 mg), and residue XII (2.448 g, fractions 86−88) was rechromatographed over Si gel and Sephadex LH-20 to give compounds **6** (13.5 mg) and **9** (7.6 mg).

19(4→3)*abeo*-8α,13(S)-Epoxylabda-4(18),14-diene (1): colorless oil; $[\alpha]^{23}_D$ +49.5° (*c* 1.05, $CHCl_3$); IR (film) ν_{max} 3078, 2932, 2858, 1643, and 888 (>C=CH_2), 1453, 1377, 1102, 1084,

1071, 963, 909, 841 cm^{-1}; ^1H and ^{13}C NMR, Table 1; EIMS *m/z* 288 (1) $[M]^+$, 273 (100) $[M - Me]^+$, 255 (27) $[M - Me - H_2O]^+$, 190.1724 (40) (calcd for $C_{14}H_{22}$, 190.1720), 175.1487 (26) (calcd for $C_{13}H_{19}$, 175.1486); HREIMS *m/z* 288.2454 (calcd for $C_{20}H_{32}O$, 288.2452).

19-*nor*-Abieta-4(18),8,11,13-tetraen-7-one (2): colorless oil; $[\alpha]^{23}_D$ +27.4° (*c* 1.13, $CHCl_3$); IR (film) ν_{max} 2918, 2950, 1682, 1607, 1566, 1493, 1464, 1444, 1415, 1383, 1257, 894 (>C═CH_2), 834 cm^{-1}; ^1H and ^{13}C NMR, Table 2; EIMS *m/z* 268 (91) $[M]^+$, 253 (47) $[M - Me]^+$, 225 (34) $[M - C_3H_7]^+$, 211 (100); HREIMS *m/z* 268.1819 (calcd for $C_{19}H_{24}O$, 268.1826).

12-Hydroxydehydroabietic acid (3): colorless oil; $[\alpha]^{23}_D$ +35.0° (*c* 2.63, $CHCl_3$); IR (film) ν_{max} 3364 (OH), 3200−2800, 1697 (COOH), 2930, 2870, 1654, 1595, 1541, 1508, 1474, 1459, 1419, 1270, 1176 cm^{-1}; ^1H and ^{13}C NMR, Table 2; EIMS *m/z* 316 (100) $[M]^+$, 301 (54) $[M - Me]^+$, 255 (64), 213 (43), 150 (17); HREIMS *m/z* 316.2038 (calcd for $C_{20}H_{28}O_3$, 316.2038).

Antitumor-Promotion Evaluation of Compounds 1−10. The inhibition of Epstein−Barr virus early antigen (EBV−EA) activation was assayed using Raji cells (virus nonproducer), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 10% fetal bovine serum−Roswell Park Memorial Institute (FBS RPMI) 1640 medium solution (Nacalai Tesque). The indicator cells (Raji) (1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of the medium containing *n*-butyric acid (4 mM, inducer) and 32 pmol of TPA [20 ng/mL in dimethyl sulfoxide (DMSO) and a known amount of test compound in DMSO]. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV−EA-positive sera from nasopharyngeal carcinoma patients and were detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated twice. The average EA induction was compared with that of positive control experiments with *n*-butyric acid plus TPA in which EA induction was ordinarily around 30%.

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